CONTRIBUTIONS TO OUR KNOWLEDGE OF THE ACTINOMYCETALES. I.

A Case of Hereditary Variation in the Genus Actinomyces.

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(Plate iii; eleven Text-figures.)

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In a microbiological analysis of a soil from a lawn in the grounds of Sydney University in search of organisms of the genus *Micromonospora* (Ørskov, 1923; Jensen, 1930) the writer isolated an organism which seemed difficult to locate, since it exhibited characters belonging to both of the genera *Actinomyces* and *Mycobacterium*, which are not very well separated from each other. In quite young cultures it formed branching filaments, which soon broke up into short, rod-shaped, partly acid-fast elements; in this stage the organism could not be distinguished from the ordinary saprophytic mycobacteria (*Myc. phlei*, *lacticola*, etc.). In some instances, 4–5 weeks old cultures produced spots of white aerial mycelium, like that which is characteristic of most actinomycetes; by plating from this mycelium, cultures were obtained of an organism, which consisted of a richly branching mycelium, entirely actinomyces-like, and without any tendency to spontaneous "fragmentation". Another organism behaving similarly was later isolated from another soil from a flower bed. It might be imagined that the cultures had, from the beginning, represented mixtures of two different organisms, but in view of the occurrence of the phenomenon in two different strains it seemed more likely to be what has been variously described as "bacterial mutation", "clone transformation", "saltation", "microbial dissociation", or "cyclogeny". Since this has an important bearing on the classification of microorganisms it was decided to subject the phenomenon to a closer study, in connection with a more general investigation of the occurrence and activities of actinomycetes in Australian soils.

Review of the Literature.

The phenomenon referred to above is intimately bound up with the whole problem of microbial variation and life cycles in bacteria. Since the literature on this subject is too vast to be reviewed here (for references, see Löhnis, 1921, Hadley, 1927, and Arkwright, 1930), we will deal only with the contributions referring to hereditary changes in the genera *Corynebacterium*, *Mycobacterium*, and *Actinomyces*. Metchnikoff (1888), Bruns (1895) and Coppen-Jones (1895) noted the occurrence of long, sometimes richly branching filaments in old cultures of the tubercle bacillus, which led them to regard this organism as a developmental stage of a more highly differentiated fungus. A permanent transformation to such a form was, however, not observed, and would indeed have met with little

confidence in that era of monomorphistic views. Kitt (1897) found an actinomyces-like organism arising in broth-cultures of *Bact. erysipelatos suum*, of which he believed it to be a developmental form; later, however, he revoked his first statement, attributing it to an error caused by a "tenacious symbiosis" between the bacterium and a contaminating actinomyces; it seems difficult today to see which of these alternatives is correct. Two interesting contributions were furnished by Cache (1901) and Spirig (1903). They found in old cultures of *Corynebact. diphtheriae* a filamentous organism, which could be isolated, and appeared like an actinomyces. Spirig observed a formation of aerial mycelium in this organism and succeeded in carrying it back to a morphological stage resembling the culture in which it had originally arisen. The proof of its genetic connection with *Corynebact. diphtheriae* was complete neither in Cache's nor in Spirig's work, and their statements seem therefore to have received little attention, and have remained unconfirmed. Since these organisms grew for many generations as actinomycetes, they were obviously different from the filamentous, branching forms occasionally seen in old cultures of *Corynebact. diphtheriae*. Abbott and Gildersleeve (1904) found these forms occurring so rarely and inconstantly that they considered it justifiable to regard them as "involution forms", and Martin, Loiseau and Gidon (1924) were able to produce them experimentally in great abundance in one particular strain of *Corynebact. diphtheriae* by growth in broth under reduced oxygen tension; when transferred to serum, they reverted at once to the normal type; a hereditary change had thus not taken place here. This seems to occur in *Corynebact. murisepticum* (Holzhausen, 1926), which can appear either as short rods or as long filaments; these characters remain constant on agar media, but tend to revert into each other in broth culture.* Brulowa (cit. by Kedrowsky, 1928) is said to have transformed *Corynebact. diphtheriae* into an actinomyces-like organism by treatment with radium rays. Finally, a dissociation into "smooth" and "rough" variants, not connected with profound morphological changes, has been observed in *Corynebact. diphtheriae* (see Arkwright, 1930). In other corynebacteria, complex life-cycles have been described by de Negri (1916) and Mellon (1920, 1926); the latter author claims to have stabilized several stages of this cycle. Gildemeister (1921) found in the organism of tuberculosis in turtles (*Mycobact. chelonei*) a dissociation into two varieties, the "normal" forming flat, dry colonies with a rough surface, and constantly splitting off the "variant", which forms soft, raised colonies with a smooth surface; this latter kept fairly constant, but showed a reversion to the "normal" in subcultures from very old broth cultures. Vaudremer (1921) found that certain strains of *Mycobact. tuberculosis* lost their acid-fastness when grown on dextrose-agar or agar without glycerin; after having undergone this treatment they produced actinomyces-like forms when grown in peptone-solution; the original form could be regenerated by culture on serum-containing media or on glycerinated potatoes. Kedrowsky (1928) claims to have stabilized an actinomyces-like phase of growth in *Mycobact. tuberculosis* as well as in *Myc. leprae* (see below), and the same statement concerning the former organism is made by Karwacki (1929). It has been known for a long time that *Myc. lacticola* exhibits two cultural varieties, α *planum* and β *perrugosum*. Haag (1927) showed, on the basis of a study of a large number of strains, that these two forms show many intergradations, and that the "perrugose" variety can be transformed experi-

* It may be questionable, however, whether this is really a Corynebacterium.

mentally into the "plane" by growth on agar with addition of old cultures (accumulation of metabolic products). Also spontaneous, mutation-like changes took place. Two strains of the "plane" variety produced secondary colonies of a type described as *Mycobact. eos* (Büttner, 1926), and a case was observed, where a perrugose strain spontaneously produced a plane variant. *Myc. phlei* had likewise a perrugose and a plane form, the latter sometimes being produced by dissociation from the former. One case of variation is particularly interesting, since it seems to represent the origin of an actinomyces as a variant from a mycobacterium. Unfortunately, Haag's description of the phenomenon is very brief, and runs thus: "Merkwürdig verhält sich Stamm 73, welcher zunächst als *Mycobact. phlei* festgestellt war, dann unter Abspaltung eines *phlei*-Stammes (73a) braungelb, matt, körnig und knorpelig wurde, also aktinomyzetenähnliches Wachstum annahm". The existence of an actinomyces-stage in the life-cycle of *Mycobact. leprae* has frequently been alleged. Deycke and Reschad (1905) isolated from a leprome an actinomyces which they believed to be genetically connected with *Myc. leprae*. Kedrowsky (1910) obtained similar results and stated that an organism, isolated from leprous lesions and capable of producing morbid affections in rabbits, had a complex life-cycle comprising acid-fast rods, non-acid-fast rods, and non-acid-fast actinomyces-forms. Similar results have been found by Bayon (1912), Johnston (1917), and others. Duval and Harris (1913) found *Myc. leprae* constantly acid-fast, and believed the aberrant forms to be contaminations. Recently Kedrowsky (1928) has reviewed the question in an interesting paper in which he states that subcultures from very old cultures of *Myc. leprae* and *tuberculosis* are rich in long, branched elements which have partly lost their acid-fastness; by further culture in rich media they soon regain their normal appearance and acid-fastness, but in media poor in nutrients, regular actinomycetes arise as fringes around the colonies. Kedrowsky argues, therefore, that both *Myc. leprae* and *Myc. tuberculosis* should be classed with the genus *Actinomyces*. In this last genus, which is notorious for the instability of its characters, the observations concerning hereditary variation are numerous. Pellegrino (1906) claimed to have transformed an actinomyces into a mycobacterium-like organism by culture in sterile butter; the actinomyces-form could be regenerated by culture on potato. Souza-Araujo (1929) reported a similar case: a true actinomyces isolated from leprous lesions could by culture on fat-containing media be transformed into acid-fast rods, said to be constant. Kedrowsky (1928) mentions the possibility of transforming actinomycetes into diphtheroid organisms by culture in media very rich in nutrients, and Sanfelice (1921, 1924) claims to have transformed two different actinomycetes into *Myc. tuberculosis*-like organisms through animal-passage. Lieske (1921) mentions several cases of mutation-like phenomena in actinomycetes—loss of spore formation, changes in pigmentation, etc. Among other things he (like Ørskov, 1923) observed a formation of a yellow variant from the normally red *Act. polychromogenes*, and a gradual transformation of an anaerobic, short-hyphed form into an aerobic, long-hyphed form. Finally, Ørskov (1923) observed cases where actinomycetes gradually abandoned their mycelial growth and passed into stages where they were indistinguishable from corynebacteria or mycobacteria. A similar case was observed by Abramow (1912) in an actinomyces isolated from meningitic pus; it produced (*a*) branching rods of a diphtheroid type and (*b*) long, richly branching filaments, which formed dry colonies, adhering to the substratum; this latter type changed into the former after a few transfers.

Morphological and Cultural Characters.

As mentioned above, two strains of organisms were isolated from two different soils. They are here termed I and II, the affixes R and F signifying, respectively, the original rod-shaped, mycobacterium-like form, and the modified, filamentous, actinomyces-like form. Single-cell cultures of the two R-forms were obtained by means of the method of Ørskov (1922), and these cultures were examined on the following media:

Dextrose-asparagine-agar: Dextrose 10·0 gm.; asparagine 1·0 gm.; K_2HPO_4 0·5 gm.; agar 15·0 gm.; H_2O 1,000 c.c.

Dextrose-asparagine-solution: Same without agar.

Dextrose-nutrient-agar: Dextrose 10·0 gm.; meat extract 5·0 gm.; peptone 10·0 gm.; NaCl 5·0 gm.; agar 15·0 gm.; H_2O 1,000 c.c. pH 7·0.

Dextrose-broth: Same without agar.

Plain nutrient agar and broth: Same two media without dextrose.

Gelatin: Plain (15% gelatin in tap water) or corresponding to nutrient agar; pH 7·0.

Potato plugs.

Milk.

Cultures were incubated at 30–32° C. and at room temperature (20–22° C.).

During the course of the investigation, several modified types, besides the F-forms, were isolated. A list of these is given below.

Designation.	Appearance.	How obtained.
Original forms:		
IRs	Rod-shaped, producing soft red growth.	From single cells of original cultures isolated from soil.
IIRs	Rod-shaped, producing soft red growth.	
Modified forms:		
IRh	Rod-shaped with tendency to filament-formation, producing hard pink growth.	Arises spontaneously in cultures of IRs, and can be produced experimentally.
IRy	Rod-shaped, producing soft yellow growth.	Produced by exposure of IRs to ultra-violet radiation.
IRw	Rod-shaped, producing soft white growth.	
IIRh	Rod-shaped, producing hard pink growth.	Arises spontaneously in cultures of IIRs, and can be produced experimentally.
IF	Filamentous, producing firm pink growth with aerial mycelium.	Arises spontaneously in cultures of IRs.
IFy	Filamentous, producing firm yellow growth with aerial mycelium.	Arises spontaneously in cultures of IRy.
IFw	Filamentous, producing firm white growth with aerial mycelium.	Arises spontaneously in cultures of IRw.
IIF	Filamentous, producing firm pink growth with aerial mycelium.	Arises spontaneously in cultures of IIRs.

Form IRs.

Dextrose-asparagine-agar: At room temperature the growth on agar slopes is restricted, raised, with smooth, shiny surface and flat myceloid edges, colour* after 15 days Begonia Rose to Rose Doree (Rdg. I, 1b–I, 30–Rb). The consistency is after 3–4 days very soft and moist, after 1–2 weeks tough and pasty. Small specks of white aerial mycelium are usually seen after 2–4 weeks; they represent the beginning of the IF-form. Occasionally hard granules of the type IRh appear, and the culture may on transfer change as a whole to this type. At 30–32° C. the growth has a rugose surface and a paler pink colour, after 3–4 weeks changing to dull brownish-grey, with some soluble pigment of the same colour.

Dextrose-asparagine-solution: At room temperature or at 30–32° C. growth starts as small pale-rose flakes in the solution; after 12–15 days voluminous pink sediment and soft, silky, pale-rose surface pellicle; after 20 days at room temperature the pellicle is very thick, soft, colour as on agar.

Dextrose-nutrient-agar: Growth at room temperature is abundant, raised, restricted, after 3–4 days soft and smooth, with myceloid edges, after 1–2 weeks with coarsely folded surface; colour after 16 days Coral Red (Rdg. XIII, 5'OOR). Light-brownish soluble pigment and a few colonies of IF are seen after 3 weeks. Similar growth at 30–32° C.; the F-form is not observed here.

Nutrient agar: Scant, uncharacteristic growth, restricted, orange-red. No pigment. The F-form arises in old cultures.

Dextrose-broth: After 3–4 days slight turbidity, small orange flakes on bottom of tube; after 2–3 weeks orange flaky sediment, no surface growth; broth clear, becomes turbid when shaken. Thick red surface pellicle in older cultures. No acid-formation.

Gelatin (plain or nutrient, at room temperature): Thin yellowish growth along stab, with short filaments radiating into gelatin; flat, red, wrinkled surface-colony. No pigment. No liquefaction after 6–8 weeks.

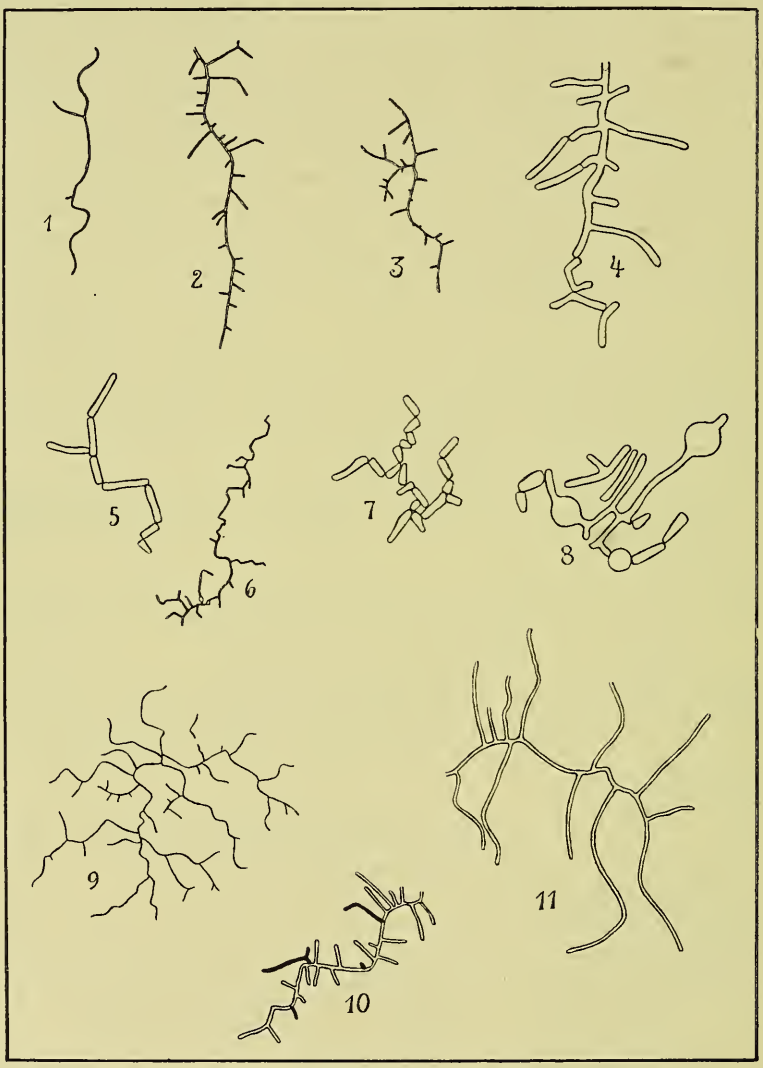
Potato: At room temperature after 3–4 days smooth, soft, pink growth, gradually becoming deeper red and wrinkled. After 2–3 weeks numerous spots of white aerial mycelium (IF), and sometimes raised, hard, pale-pink granules of the type IRh. At 30–32° C. similar, but the spots of aerial mycelium seem to arise less frequently.

Milk: Growth starts as small orange granules on surface; after 1–2 weeks they fall to the bottom and form a thick, soft, orange-red sediment. The milk is not coagulated or hydrolyzed; in quite old cultures (10–12 weeks at room temperature) it becomes viscous and semi-transparent.

Morphology.—Direct observation of the growth (Ørskov, 1923) on blocks of dextrose-agar shows the following mode of development. After 20–24 hours at room temperature the cells of the inoculum have germinated and formed a small mycelium of fairly long, wavy hyphae, 0·4–0·5 μ thick, extending to a length of 70–100 μ, and producing a few side branches (Text-fig. 1). After 2 days these mycelia reach a considerable extent; hyphae produce numerous rectangularly situated side branches, and show some tendency to penetrate into the medium. Aerial hyphae are formed (only visible under the microscope), arising as small refractive granules in the substratum mycelium, and gradually stretching into short, somewhat irregular threads; when examined under immersion lenses, these

* Description of colours here and in the following after Ridgeway, Colour Standards and Nomenclature.

aerial hyphae are not different from the substratum hyphae. Septa are not visible in the substratum mycelium, and the culture makes at this stage a perfectly actinomyces-like impression (Text-fig. 2–3). When the growth is scraped off and examined in ordinary smear-preparations, only slender, branching rods of varying



Text-figures 1-11.

1.—IRs. Dextrose-asparagine-agar, 1 d. room tpt. (living material). 2.—Same, 2 d. 3.—Same, seen under high-power dry lens; aerial hyphae deeply shaded. 4.—Same, 3 d. Immersion lens. 5.—IRh. Dextrose-nutrient-agar, 3 d. room typt. 6.—IRs. Dextrose-asparagine-agar, 5 days room tpt. 7.—Same, 6 d. 8.—Same, potato-extract-agar, 5 d. 32° C. 9.—IF, Dextrose-asparagine-agar, 2 days room tpt. 10.—Same, 3 d., seen under high-power dry lens; aerial hyphae heavily shaded. 11.—Same, under immersion lens. Magnifications: Figs. 1, 2, 3 and 6, × 500; Figs. 4, 5, 7 and 8, × 2,000; Fig. 9, × 250; Figs. 10 and 11, × 1,000.

length, 4–10 × 0·3–0·5 μ, are seen (Plate iii, fig. 3); in carefully made impression-preparations one may occasionally see the true mycelia, but mostly only irregularly staining rods, often in V- or Y-shaped arrangement. After 3 days the organism passes into another stage of growth. Septa are now formed in the substratum mycelium (Text-fig. 4), cutting off rod-shaped cells which occupy an angular position, adjoining each other at the corners like diphtheroids. In the following days this process of septation and of multiplication by cell-division goes on (Text-figs. 6–7). After 6 days all mycelial growth has disappeared.* After 14 days the appearance is unaltered; the colonies consist exclusively of short, unbranched cells, 0·5–0·6 × 1·5–2·0 μ, always in angular arrangement (Plate iii, fig. 4); in still older cultures almost coccoid cells are formed (Plate iii, figs. 5, 6, 9). Thus, the mycelial growth, with which the development of the colony starts, is after a few days superseded by cell-division of the "snapping" type characteristic of corynebacteria (Graham-Smith, 1907); there is never any indication of the "slipping" or "gliding" growth which characterizes *Myc. tuberculosis* (Miehe, 1910) and several saprophytic mycobacteria (Haag, 1927). The aerial hyphae disappear after some days; they appear to fall down and become indistinguishable from the substratum hyphae. The course of development is entirely characteristic for actinomycetes of Ørskov's Group II (Ørskov, 1923).

The cells are gram-positive, but usually stain rather irregularly with anilin dyes, appearing banded and granulated, like many corynebacteria and mycobacteria (Plate iii, fig. 4). They exhibit a certain degree of acid-fastness; after staining with hot carbol-fuchsin most cells are strongly decolourized by 20% sulphuric acid, but a certain number of them retain the stain very well; this is particularly true in old cultures (Plate iii, fig. 9). On other media the appearance of the cells is similar; there is a tendency to earlier production of short, coccoid cells in rich media, such as dextrose-nutrient-agar. At 30–32° C. there is in all media a tendency to production of longer cells than at room temperature; after a few days the culture contains many swollen, fusiform to lemon-shaped cells (Plate iii, fig. 10); they are most numerous on dextrose-nutrient-agar and potato, and particularly on dextrose-asparagine-solution of pH 8·3, where the microscopic appearance often resembles a minute yeast (at pH 5·3–5·7 there is a production of longer, branched, partly swollen cells). The formation of these swollen cells is easily followed on potato-extract-agar, where they arise in great abundance; after 1–2 days there is formed the usual rectangularly branched mycelium, which after 3–4 days divides into rod-shaped cells of varying length; after 4–5 days these cells show local swellings, often measuring 2·0–2·7 μ in diameter (Text-fig. 8). The swollen cells are viable; when transferred to fresh agar, they germinate readily with the formation of a normal mycelium. Similar forms have been described in corynebacteria, mycobacteria and actinomycetes by several authors. Vallée (1903) mentions "des éléments fusiformes, dont la partie seule se colore bien" in *Actinomyces polychromogenes*, and of the same organism Lieske (1921) gives illustrations (Fig. 35, p. 64) which could very well represent the present one. Korn (1908) describes similar formations, resembling heads of spermatozoa or fragments of shattered actinomyces-granules, in *Mycobact. friburgense*. Identical-looking phenomena are described by Vierling (1922) as "involution forms" in cultures of soil mycobacteria (or actinomycetes?). Ørskov

* On the surface of the agar; in the depth the development is difficult to follow, but septation seems to take place here, too.

(1923) found a general tendency to production of such forms in a whole category of actinomycetes, and Henry (1910) found them arising in cultures of an anaerobic actinomyces when exposed to the air. Finally, Bergstrand (1923) describes similar forms under the name of "chlamydospores" in *Corynebact. lymphogranulomatis*; they show, according to his figures, a mode of formation analogous to that in our IRs, but his statement, that they multiply by yeast-like budding, suggests that they may, at least partly, be of another nature. Apparently these swollen cells represent nothing but a strongly accentuated development of the club-shaped cells often observed in corynebacteria and mycobacteria, but they are probably of another nature than the yeast-like cells described below under IIRs. The regularity of their appearance and their vitality render it unjustifiable to dispose of them as "involution forms", and their germination into a mycelium does not support the theory that they may be gonidangia. Further, their mode of formation inside a single cell shows that they are not "zygospores" arising through conjugation of two cells (Löhnis and Smith, 1923).

Form IRh ("hard" type of IRs).

Dextrose-asparagine-agar: Growth at room temperature after 3–4 days thin, flat, almost colourless, with some raised pink granules of hard consistency. After 1–2 weeks the growth is abundant, much raised and wrinkled, adhering strongly to the medium, surface cracking; consistency hard, dry and crumbly, but not cartilaginous like a true actinomyces; colour after 15 days Alizarin Pink to Venetian Pink (Rdg. XIII, 1'd.–XIII, 1'f.), paler than IRs. A faint pinkish-grey soluble pigment is formed after 3 weeks. At 30–32° C. similar, growth more dirty pinkish-grey; scant white aerial mycelium may be seen, but the F-form does not arise.

Dextrose-asparagine-solution: Growth as hard granules on bottom and along surface of solution; tendency to formation of a tough, granulated, pink surface pellicle.

Dextrose-nutrient-agar: Growth at room temperature abundant, much raised and granulated, of a crumbly consistency, but not so hard as on dextrose-asparagine-agar; colour after 15 days Carnelia Red (Rdg. XIV, 7'R-O). Light-brown soluble pigment after 2–3 weeks. Growth at 30–32° C. similar, but of softer consistency.

Nutrient agar: Scant, uncharacteristic growth, granulated, orange-red.

Dextrose-broth: Broth clear with granulated orange sediment; no surface growth; no uniform turbidity when the tube is shaken. No acid-formation.

Gelatin: = IRs.

Potato: Growth starts at room temperature as orange-red, raised, firm granules, gradually spreading after 1–2 weeks, becoming deeper red; consistency partly crumbly, partly soft after 3 weeks. No aerial mycelium. At 30–32° C. similar growth, deeper red.

Milk: Orange granules on surface. Milk somewhat thickened after 3–4 weeks at 30–32° C.

The F-form has never been seen to arise in cultures of this type.

Morphology.—The growth on dextrose-asparagine-agar starts in the same manner as IRs, but there is a much more pronounced tendency to growth in the

depth of the medium, and much less tendency to septation. After 14 days at room temperature the elements are still chiefly filamentous and non-septate, only here and there showing rod-shaped cells in angular arrangement. On dextrose-nutrient-agar septation sets in after 3 days at room temperature, and the culture appears here much like IRs, although the cells are generally longer and more slender, 2·0–8 × 0·4–0·5 μ (Text-fig. 5). In smear preparations, especially from dextrose-asparagine-agar or solution, the long, non-septate filaments are very frequently seen, but they seem very easily to be broken up by rough treatment of the material (Plate iii, fig. 2). The rod-shaped cells are always longer, more slender and more irregular in shape than those of IRs. They are also less acid-fast; none of them retains the dye perfectly, but they show a number of acid-fast granules. At 30–32° C. swollen cells, similar to those of IRs, are formed.

Form IRy (yellow modification of IRs).

Dextrose-asparagine-agar: Growth at room temperature restricted, raised, with smooth, shiny surface and flat, myceloid edges; consistency soft and moist, pasty. Colour after 16 days Ochraceous Salmon to Flesh-Ochre (Rdg. XV, 13'OYO-XIV, 9'OR-Ob).

Dextrose-asparagine-solution: At room temperature a thick soft pellicle of same colour as on agar, and thick yellow sediment. Solution clear, becomes turbid on shaking.

Dextrose-nutrient-agar: Good growth at room temperature, raised, soft, smooth, with myceloid edges; colour after 16 days Apricot Orange (Rdg. XIV, 11'). Growth somewhat granulated and folded after 3 weeks, with a faint brown soluble pigment.

Dextrose-broth: Broth slightly turbid after 4 days at room temperature, with yellow flakes on bottom. After 1–2 weeks thick yellow sediment, no surface growth; broth almost clear, turbid when shaken. Yellow pellicle in older cultures.

Gelatin: Scant yellow growth along stab and on surface; short filaments radiating from stab; no liquefaction.

Potato: Soft, smooth, raised, yellow-ochre growth after 4 days at room temperature. After 2–3 weeks numerous hard granules with white aerial mycelium arise, representing the form IFy.

Milk: Growth starts as small loose yellow flakes along the wall of the tube, after 7–8 days settling and forming a soft yellow sediment; milk unchanged after 4 weeks at room temperature.

Morphology.—This organism corresponds in morphological respect perfectly to IRs, but the formation of a "hard" variant, corresponding to IRh, has not been observed.

Form IRw (white modification of IRs).

Dextrose-asparagine-agar: Good growth at room temperature, raised surface, first smooth, later roundly-folded, edges slightly myceloid; consistency soft and pasty; colour milk-white. After 4–5 weeks a faint pink colour develops, but platings show only white colonies.

Dextrose-asparagine-solution: Thick, soft, white pellicle and sediment after 3 weeks at room temperature; solution clear, becomes turbid when shaken.

Dextrose-nutrient-agar: Growth similar to that on dextrose-asparagine-agar, but surface after 10–12 days more wrinkled and granulated on central part, edges entire. Faint brown pigment after 2–3 weeks.

Dextrose-broth: Slight turbidity after 4 days, white flaky sediment. After 2 weeks abundant white sediment and broken white scum on surface. Broth clear, turbid when shaken. No acid after 3 weeks.

Potato: Scant, smooth, raised, white growth after 4 days at room temperature. After 1–2 weeks growth somewhat better, with folded surface, else unchanged. Small specks of white aerial mycelium (Fw) arise after 3 weeks.

Gelatin: Thin white growth along stab, with short, horizontally radiating threads. White surface growth. No liquefaction.

Milk: Growth starts as small white flakes along wall of tube, later (1–2 weeks) forming a white sediment. Milk unchanged.

Morphology.—Exactly like IRs and IRy. These three forms are distinguished from each other practically only by the pigmentation; the formation of the yellow and the white variety from the original red seems analogous to the well-known production of a lemon-yellow and a white variety (known respectively as *Staphylococcus citreus* syn. *Micrococcus pyogenes β citreus*, and *Staphylococcus albus* syn. *Micr. pyogenes γ albus*) from the orange *Staph. aureus* syn. *Micr. pyogenes α aureus* (Neumann, 1897), only in the staphylococci these phenomena occur spontaneously, while in the present instance they were only observed after treatment with ultraviolet rays.

Form IIRs.

Dextrose-asparagine-agar: Growth after 6 days at room temperature restricted, raised, with rugose surface and myceloid edges, consistency soft and moist. After 2 weeks the growth is abundant, surface much wrinkled, consistency tough-pasty, colour like IRs. Specks of white aerial mycelium (IIF) arise after 3–4 weeks, sometimes earlier. At 30–32° C. the growth is paler pink, after 3 weeks dirty pinkish-grey, with some soluble pigment of the same colour.

Dextrose-asparagine-solution: Soft, silky, pink pellicle and sediment; solution clear.

Dextrose-nutrient-agar: After 1 week at room temperature similar to dextrose-asparagine-agar, but better growth, of a deeper red colour. After 15 days abundant, much raised and folded, Grenadine Red to Flame Scarlet (Rdg. II, 7R-O-9OR-O). Faint brown soluble pigment after 3 weeks. At 30–32° C. the growth is hardly different, develops somewhat more rapidly.

Dextrose-broth: Flaky orange sediment after 1 week at room temperature: similar, more voluminous, after 2–3 weeks. Broth clear, only occasionally with granules of surface growth. At 30–32° C. similar growth. No acid is formed. Thick red surface pellicle in older cultures.

Gelatin: At room temperature, 3–5 weeks, filiform growth along stab, with short radiating threads, and wrinkled red surface growth. No liquefaction.

Potato: After 1 week at room temperature soft, moist, glistening, red growth; after 2 weeks round-granulated surface, pasty consistency, intense red colour. Numerous specks of white aerial mycelium (IIF).

Milk: Small reddish flakes swimming on surface and attached to the tube after 3–6 days; after 2–3 weeks a flaky, red sediment is formed. Milk remains unchanged.

Morphology.—The development on dextrose-asparagine-agar takes place in the same manner as in IRs. The rod-shaped cells are generally somewhat longer and more slender and show less tendency to production of swollen, lemon-shaped forms at 30–32° C., particularly in dextrose-asparagine-solution, where the shape of the cells is little influenced by the reaction, unlike IRs. The cells are also less acid-fast than those of IRs; on dextrose-asparagine-agar there is almost no acid-fastness, but on dextrose-nutrient-agar this strain is like IRs. In dextrose-broth (to a smaller extent also on dextrose-nutrient-agar) there is frequently after 4–6 days at 30° C. a formation of remarkable, big, oval, yeast-like cells, measuring 4–5 μ, usually aggregated in small clumps, containing a coarsely granulated cytoplasm, and staining intensely with carbol fuchsin and Delafield's haematoxylin (Plate iii, figs. 7, 8). They seem to correspond to similar phenomena observed in corynebacteria by de Negri (1916), Mellon (1920), and Bergstrand (1922), and in actinomycetes by Leyton and Leyton (1916), who call them "megaspores". Similar things have been described as "gonidangia" in other bacteria by Löhnis and Smith (1923), Oesterle and Stahl (1929), Gibson (1928), and several others.* The gonidangium-nature of the present cells is uncertain, since it has not been found possible to follow their further development when transferred to fresh media; in hanging agar-block preparations they remain unchanged for up to 10–12 days, while the normal rod-shaped cells multiply vigorously. Neither have they shown any development in hanging-drop preparations (dextrose-broth) for up to 3 days. In preparations from broth-cultures these cells often show evidence of a reproduction by yeast-like budding (Plate iii, fig. 8), as described by Bergstrand (1923), whose "chlamydospores", however, seem partly identical with the swollen forms in our IRs. These latter are different from the yeast-like cells, since they, as stated above, germinate readily on agar media. The true nature of the yeast-like cells must, therefore, for the present, be regarded as unknown. Besides these there is often after 5–6 days in broth cultures a quite abundant formation of clumps of amorphous, granulated matter, resembling the phenomenon described as formation of "bacterial plasmodia" (Almquist, 1917) or "symplasm" (Löhnis, 1921, Löhnis and Smith, 1923). These flakes of amorphous material can quite easily be traced in hanging-agar-block preparations, like the yeast-shaped cells, and like these they remain unchanged for many days, until they are overgrown by colonies originating from neighbouring normal cells. A regeneration of cells inside them has never been observed, and there is thus so far no evidence that they represent living matter (cf. Bergstrand, 1923).

Form IIRh ("hard" type of IIRs).

Dextrose-asparagine-agar: Growth after 6 days at room temperature much raised and wrinkled, pale rose-pink, of a dry and crumbly consistency, somewhat adhering to the agar, after 12–15 days still more wrinkled. Colour Eosine Rose to Begonia Pink (Rdg. I, 1d–I, 1b). The aerial mycelium is often macroscopically visible as a thin white veil all over the growth.

Dextrose-nutrient-agar: Growth after 6 days at room temperature similar to dextrose-asparagine-agar, but more red; after 12–15 days abundant, much

* The *Schizosaccharomyces filtrans* described by Lewis (1927) is probably also an organism of this group, and no yeast at all.

folded, of a somewhat pasty consistency; colour Light Coral Red (Rdg. XIII, 5'00-Rb). Light-brown soluble pigment after 3 weeks.

Dextrose-broth: Abundant growth after 3 weeks at room temperature; granulated red sediment and thick, easily broken, red pellicle. No acid.

Potato: Growth at room temperature first granulated, pale-orange, of a firm consistency, after 3–4 weeks dull red with thin veil of white aerial mycelium on upper dry part.

Milk: Small red granules on surface, partly falling to the bottom. Milk unchanged after 4 weeks.

At 30–32° C. the cultures are hardly distinguishable from IIRs.

As in the case of IRh, an F-form has not been seen to arise.

Morphology.—Microscopically this strain can hardly be distinguished from IIRs, except for the more pronounced tendency to formation of aerial hyphae.

Form IF (filamentous form of IRs).

Dextrose-asparagine-agar: Growth at room temperature after 5 days consists of small, round, raised, partly confluent, pale-pink granules with myceloid edges, growing deeply into the agar and covered by a thin white aerial mycelium. After 12–15 days a granulated, confluent growth of a tough, cartilaginous, entirely actinomyces-like character, adhering strongly to the medium; colour Orient Pink (Rdg. II, 9 OR-Of). Similar at 30° C. The aerial mycelium is rather variable on this medium, sometimes almost absent, sometimes, particularly at room temperature, forming a well-developed, white, felty layer. The consistency remains cartilaginous in very old cultures (5–6 months).

Dextrose-asparagine-solution: Growth starts as small colourless granules on surface and bottom of solution, after 12–15 days, forming a thick, granulated, pale rose pellicle. Solution remains perfectly clear.

Dextrose-nutrient-agar: After 5 days at room temperature small pinkish-orange granules without aerial mycelium; after 12–15 days raised, round, partly confluent granules, with scant white aerial mycelium; consistency hard and cartilaginous; colour Grenadine Pink (Rdg. II, 7R-Od). Faint brown soluble pigment. Similar growth at 30° C.; after 3–4 weeks the central superficial part of the growth assumes a more loose and crumbly consistency.

Dextrose-broth: Small pink granules on surface, after 12 days at room temperature forming a coherent, pale-pink pellicle with thin white aerial mycelium. Broth remains perfectly clear. No acid is formed.

Gelatin: Scant filiform growth with short radiating threads along stab; rose-red surface colony with thin white aerial mycelium; no liquefaction.

Potato: Growth spreading, granulated, pink, after 12 days covered by a thin white aerial mycelium; consistency hard and cartilaginous, after 5–6 weeks at 30° C. brittle and crumbly in dried part of growth.

Milk: Small pale-pink granules on surface, gradually forming a red pellicle with thin white aerial mycelium. Milk remains unchanged.

Morphology.—The growth on dextrose-asparagine-agar starts in a manner similar to that of IRs. After 2 days at room temperature extensive mycelia are formed, measuring 100–150 μ across, consisting of richly branched hyphae with numerous aerial filaments and a marked tendency to growth in the depth of the agar; the central filaments are thicker (up to 0·8–0·9 μ) than the end

branches (0·4–0·5 μ). After 3 days the mycelia reach a very large extent and exhibit all the characters of a true actinomyces of Group I (Ørskov, 1923). In the following time the organism seems to remain at this stage of development (Text-figs. 9–11); unlike IRs, an angular arrangement is not seen, the hyphae remaining wavy and irregularly branching; septa are not formed. In the aerial hyphae a differentiation into spores, such as in Ørskov's Group I, is not seen, but when the aerial mycelium is scraped off and examined in ordinary preparations, the hyphae are easily broken into fragments of varying length, down to 5–6 μ long and 0·4–0·5 μ thick. Ordinary stained preparations show a typical actinomyces-mycelium: long, slender, branching filaments, mostly 0·4–0·5, up to 0·8–1·0 μ thick, staining rather irregularly with anilin dyes (Plate iii, fig. 1). The hyphae are gram-positive, but only slightly acid-fast; like IRh, they are mostly decolourized by 20% sulphuric acid, but show numerous acid-fast granules. The loose and crumbly growth in old cultures on dextrose-nutrient-agar and potato shows microscopically many rather short, irregular, branching rods, somewhat similar to IRh, and quite markedly acid-fast; a similar phenomenon is seen in dextrose-asparagine-solution of pH 8·3 after 3 weeks at room temperature. This, however, does not represent a reversion to the R-form, since these cells produce only the normal F-form when transferred to fresh medium. In dextrose-asparagine-solution of pH 8·3 there is also, previous to the formation of short forms, a production of remarkably curved and gnarled filaments with many short lateral branches bearing terminal swellings which stain intensely and give the organism a *Micromonospora*-like appearance (Ørskov, 1923; Jensen, 1930).

Forms IFy (filamentous form of IRy) and IFw (filamentous form of IRw).

These two organisms are parallel forms to IF, from which they differ only in colour.

Form IIF (filamentous form of IIRs).

Culturally this form is very similar to IF, from which it differs mainly in a more pronounced tendency to formation of aerial mycelium, particularly at room temperature, and in its colour. The growth on dextrose-asparagine-agar after 12–15 days is at room temperature Grenadine Pink (Rdg. II, 7R-Od), at 30° C. Pale Salmon to Seashell Pink (Rdg. XIV, 9'f–11'f), on dextrose-nutrient-agar at room temperature Grenadine Pink, at 30° C. Brazil Red (Rdg. I, 5i). Morphologically it is indistinguishable from IF.

Biological Characters.

So far as studied, the various forms seem identical in physiological respect, although a complete study of their physiology has not been carried out. They are all obligate aerobic organisms. Invertase, diastase and proteolytic ectoenzymes are not produced. Paraffin wax, benzene vapour, stearic acid, phenol, and cellulose are not utilized. The optimal temperature seems to be 25–30° C.; at 60° C. the cells are killed within 2 minutes; aerial mycelium of IF shows no higher thermoresistance than the substratum mycelium. The optimal reaction is at approximately neutral reaction. IRs, IIRs, IF and IIF were grown in dextrose-asparagine-solution, the reaction of which was adjusted to pH values between 5·0 and 8·3 by means of tartaric acid and sodium hydroxide. The following results were found after 12 days at 30° C.:

pH	Growth of			
	IRs	IIRs	IF	IIF
5·2	none	none	none	none
5·7	trace	trace	very scant	trace
5·9	very good	fair	fair	scant
6·5	excellent	good	good	fair
6·8	excellent	good	very good	fair
8·3	fair	scant	good	scant

Identity of the Organism.

The two original forms, IRs and IIRs, particularly the former, are doubtlessly identifiable with *Actinomyces polychromogenes* Vallée. The original description of this organism (Vallée, 1903) is not very complete, but it agrees with the present in the formation of long branching filaments in quite young cultures, short oval rods after a few days, fusiform cells in acid solution, and pigment of various red colours. Lieske (1921) and Ørskov (1923) have studied the same organism. Both authors record a spontaneous formation of a yellow variant, corresponding to our IRy. Their microscopic illustrations of *Act. polychromogenes* resemble the present organisms perfectly; Lieske's Figs. 24, 25, 26 and 35, and Ørskov's Figs. 40 and 42 could very well represent our IRs. Lieske also found *Act. polychromogenes* acid-fast under certain conditions. Neither Lieske nor Ørskov found, in disagreement with Vallée, any formation of aerial mycelium; this property had apparently been lost in the long period of artificial cultivation between the studies of Vallée (1903) and of Lieske and Ørskov (1921-1923). We may conclude from these data, that *Act. polychromogenes* is a species-group with a pronounced tendency to hereditary variation, some strains (e.g., that studied by Vallée, Lieske and Ørskov) producing yellow variants spontaneously, others (e.g., IRs) producing "hard" and filamentous variants spontaneously, besides white and yellow variants under certain conditions (after treatment with ultraviolet rays, as shown below), and others again (e.g., IIRs) producing only "hard" and filamentous variants spontaneously.

Various Factors that Influence the Appearance of Modified Forms.

Age of Cultures.—As shown above, the F-forms arise mostly in cultures 3–4 weeks old. Subculture on dextrose-asparagine-agar from some older cultures gave the following results:

IRs in dextrose-asparagine-solution, 21 weeks at room temperature: IRh predominant, IF also present.

IRs in milk, 20 weeks at room temperature: same.

IRs in dextrose-broth, 15 weeks at 30° C.: mixture of IRs, IRh, and IF; last form predominant.

IIRs on gelatin, 18 weeks at room temperature: mixture of IIRs and IIRh. On dextrose-asparagine-agar, 20 weeks at room temperature: same.

Ageing of the cultures does thus here, as in many other cases (Arkwright, 1921, 1930), give rise to variation (cf. also Kedrowsky, 1928, and Spirig, 1903).

Drying.—Numerous experiments by Almquist and co-workers (Almquist, 1917; Almquist and Koraen, 1918; Koraen, 1918) suggest that drying of the cultures

may markedly influence the life-cycle of various bacteria. In the present instance, no variation was found in subcultures on dextrose-asparagine-agar from dried-up cultures (4–5 weeks at 30° C.) of IRs and IIRs. Drying does thus not seem to be a special incitant to variation here; this has possibly some connection with the fact that mycobacteria and actinomycetes are as a whole very resistant to drying.

Metabolic Products.—The fact that the F-forms arise in rather old cultures suggests that the accumulation of metabolic products may have something to do with the phenomenon. In order to test this, IRs and IIRs were grown on media which had previously supported growth by the same organisms. Nadson and Adamovič (1910) could in this way induce remarkable morphological changes in *Bac. mycoides*, and Haag (1927) was, as mentioned above, by a similar method able to induce changes in mycobacteria. Other similar cases are quoted by Arkwright (1930).

Two series of experiments were carried out here:

I. Old cultures of the Rs-forms on dextrose asparagine-solution (2 months at room temperature) were boiled and filtered, and 1% dextrose, 1·5% agar and a trace of asparagine were added. IRs and IIRs were then grown on slopes of this medium at room temperature, with transfers every 10 to 14 days. The originally soft and moist growth became, after 2–3 transfers, dry and crumbly, with a wrinkled surface and covered by a thin white aerial mycelium. A complete change into the F-forms was not observed, but subcultures on ordinary dextrose-asparagine-agar from the fifth generation showed that the R-forms, originally of the s-type, had been changed into the h-type.

II. IRs and IIRs were grown in dextrose-peptone-solution (150 c.c. in 350 c.c. Erlenmeyer flasks) for 6 weeks at 30° C. The solutions were then boiled and filtered, and 1% dextrose, 0·5% peptone and 1·5% agar were added. IRs and IIRs were as before grown on slopes of the corresponding agar medium for five generations. IRs produced an abundant, smooth, pasty, red growth, occasionally producing the F-form, and in the fifth generation becoming more dry and crumbly. IIRs produced also an abundant growth, similar in all generations, raised and wrinkled, covered with a veil of aerial mycelium, but of a soft and loose consistency without any trace of the F-form. Transfers to dextrose-asparagine-agar from the fifth generation gave, as before, a growth of the h-types of both R-forms; a transfer from the first generation of IRs gave the normal s-form. Here again a change of the s-types into the h-types has been effected.

Ultraviolet Radiation.—In the experimental study of heredity, much attention has in recent years been paid to the influence of short-wave radiation (ultraviolet radiation and X-rays) on the progeny of irradiated organisms. In microbiology a few cases of hereditary variation due to this cause have been recorded. Henri (1914) exposed *Bac. anthracis* to ultraviolet radiation and obtained in this way three modified forms, two of which proved constant for many generations. Enderlein (1925) states that exposure to sunlight exerts a strong influence on the alleged cyclogeny of *Vibrio cholerae*, giving rise to formation of filterable gonidia and to sexual reproduction. Following up the suggestions of Enderlein, Oesterle and Stahl (1929) were able to produce filterable forms and other aberrant types of *Bac. mycoides* by exposure to sunlight or ultraviolet rays. Nadson and Philippow (1929) induced mutation-like changes in the fungus *Zygorhynchus Moelleri* and in yeasts by treatment with X-rays. The changes mentioned by Brulowa (Kedrowsky, 1928) have been referred to above.

An experiment on the influence of ultraviolet radiation on IRs and IIRs was carried out. Since no quartz-lamp was available, an arc-lamp with iron electrodes, consuming a current of 5·2 Amp., 112 V., was used as source of ultraviolet rays The material to be treated consisted of 1·5 c.c. portions of suspensions of 8 days old dextrose-asparagine-agar cultures in sterile 0·85% NaCl solution, placed in small transparent silica test tubes. These tubes were placed in a distance of 30 cm. from the naked arc, at which distance the heat effect was negligible, and subjected to the radiation for ½, 1, 2, 3, 4 and 6 minutes. After treatment, a loopful of each suspension, as well as of untreated control suspension, was transferred to slants of dextrose-asparagine-agar, and duplicates were incubated at room temperature. The control tubes of IRs showed only the normal IRs. Thirty seconds' irradiation caused a slightly delayed start of the growth, which contained numerous hard granules of the IRh-type. Irradiation for 1 to 6 minutes caused a continually thinner and more slowly starting growth, always consisting of a mixture of the soft, red colonies of IRs and the hard, raised, pale-pink colonies of IRh; most of the tubes also contained a few of the white colonies of IRw, and in one of the tubes from 3 minutes irradiation a colony of IRy appeared. Of the F-form, only a single colony was observed. It was thus possible to produce three modified forms (IRh, IRw, and IRy, although the last was very rare) by treatment of IRs with ultraviolet rays. IIRs produces a similar mixture of IIRs and IIRh, but this experiment was less convincing, since the h-type was present also in the control, although it seemed more abundant in the cultures from irradiated inoculum.

Other Factors.—Addition of 0·1% coffein or 1·0% lithium chloride (compounds which are both capable of exerting a strong formative influence on many bacteria) to dextrose-asparagine-agar had no clear effect on IRs and IIRs. The former compound did not affect the growth visibly, the latter suppressed it almost entirely. Neither did growth for 14 days on a starvation medium (pure agar in tap water) exert any influence on the subsequent growth of IRs, IRh, IIRs and IIRh on dextrose-asparagine-agar.

The experiments thus show that we can in several cases experimentally change the Rs-types into other types, but the appearance of the F-forms seems to occur quite spontaneously and is not influenced by any of the factors tested here.

Discussion.

The question of the nature of these modified forms now suggests itself. The "soft" and "hard" types of the R-forms represent probably cultural varieties analogous to the "plane" and "perrugose" varieties in the closely related mycobacteria; they may possibly also be compared with the "smooth" (S) and "rough" (R) variants of intestinal bacteria (Arkwright, 1921, 1930), though it has not been tested whether they are serologically different from each other. IRy and IRw are obviously colour-varieties of IRs, comparable to those in the staphylococci, as mentioned above. The appearance of the F-forms seems to present a more extraordinary phenomenon. The whole morphological character of the F-forms would lead us to regard them as stabilizations of the initial mycelial stage of the R-forms, but they are apparently not formed directly from these initial mycelia, since they arise only in comparatively old cultures, where all mycelial growth has disappeared long ago. What seems to happen is, that now and again a cell of the R-form reverts to the original actinomyces-like mode of growth and remains at this stage, due to causes which at present we cannot ascertain. The "hard" types

of the R-form (particularly IRh) seem to represent an incomplete step in the direction of the F-form. Whether we should regard the F-forms, which so far have proved constant, as simple hereditary variants or as stages in the life cycle of the organism can only be ascertained when the F-forms have been observed for a very large number of generations, in order to see whether they will again change back into the R-form.

The origin of the F-forms throws an interesting light upon the alleged production of actinomyces-like forms in *Corynebact. diphtheriae* (Cache, Spirig) and *Myc. leprae* and *tuberculosis* (Kedrowsky, and others). When typical actinomycetes (the F-forms) can arise in single-cell cultures of mycobacterium-like organisms (the R-forms), it would seem likely that the same phenomenon might occur in true corynebacteria and mycobacteria, from which our R-forms differ only in the extent of their initial mycelium. This difference is one of degree only, since also corynebacteria and mycobacteria may occasionally show indications of a mycelial growth (Lepeschkin, 1904, Ørskov, 1923, and Haag, 1927; Lepeschkin's *Bacillus Berestnewii*, which showed an hereditary tendency to branching and occasionally to formation of small mycelia, was in all probability a corynebacterium or a non-acid-fast mycobacterium. Haag describes, in his Fig. 2, such a case as "aussergewöhnliche Form").

The phenomenon has also an important bearing on the systematics of the genus *Actinomyces*. Ørskov (1923) suggested a division of this into two genera, primarily on the basis of the formation of septa in the mycelium. The present results show that at least this criterion cannot be used unreservedly, since on the basis of this alone we would have to place the R-forms and the F-forms in two different genera—a procedure which one cannot but feel as unnatural, despite the fact that all classification is artificial and all limits arbitrary. On the other hand, a careful study of phenomena such as these will enable us to place the systematics of the actinomycetes, as well as of other microorganisms, on a firmer and more natural basis than previously (cf. Löhnis and Smith, 1923).

Summary.

Single-cell cultures of two strains of a soil actinomyces, probably identical with *Act. polychromogenes* Vallée, produced two different growth forms. The first and original form "R" (rod-shaped) forms initially a small unicellular mycelium which soon divides into bacteria-like elements; these multiply by cell division in the manner characteristic of corynebacteria. This R-form has two subtypes; the s-type ("soft"), which is the original, produces a soft, pasty growth of a red colour; the bacteria-like elements are usually short, blunt, little branched, and partly acid-fast. The h-type ("hard") produces a dry, crumbly growth, adhering firmly to the medium and consisting of longer and more slender cells, less acid-fast than the s-type and with a marked tendency to formation of long filaments; this type arises spontaneously in cultures of the s-type and can also be produced experimentally from this. Exposure to ultraviolet rays gave rise to a yellow and a white variety of the s-type. The difference between the s- and h-types was more pronounced at room temperature than at 30–32° C. and more pronounced in one strain than in the other. These s- and h-types seem to correspond to the "plane" and "perrugose" variants of mycobacteria, and are possibly also comparable with the "smooth" and "rough" variants known to occur in numerous other bacteria. The second form "F" (filamentous) represents a stabilization of the initial mycelial stage of the R-form. It is a perfectly actinomyces-like organism,

consisting of long, delicate, branching hyphae, with a well-developed aerial mycelium, and without any tendency to divide by septa into bacteria-like elements. It arises spontaneously in old cultures of the R-form of the s-type (not in the h-type), and its appearance does not seem to be influenced by external factors.

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EXPLANATION OF PLATE III.

Fig. 1.—IF. Dextrose-asparagine-solution, 9 d. room typt. (Carbol-fuchsin). Fig. 2.—IRh. Dextrose-nutrient-agar, 12 d. room tpt. (Carbol-fuchsin). Fig. 3.—IRs. Dextrose-nutrient-agar, 1 d. room tpt. (Carbol-fuchsin). Fig. 4.—Same. Dextrose-asparagine-agar, 12 d. room tpt. (Carbol-fuchsin). Fig. 5.—Same. Potato, 13 d. room tpt. (Nigrosin). Fig. 6.—IIRs. Gelatin, 32 d. room tpt. (Nigrosin). Figs. 7, 8.—Same. Dextrose-broth, 4 d. 32° C. (Carbol-fuchsin). Fig. 9.—IRs. Dextrose-asparagine-agar, 2 months room tpt. (Hot Carbol-fuchsin, differentiated with 20% H_2SO_4). Fig. 10.—Same. Potato, 13 days 32° C. (Nigrosin).—All photographs are taken with the Zeiss "Phoku" apparatus × Apochr. 1·5 mm. N. Ap. 1·30. Magnifications × 750.